(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
25 July 2002 (25.07.2002)

PCT

(10) International Publication Number
WO 02/057301 A2

(51) International Patent Classification[7]: C07K 14/005

(21) International Application Number: PCT/GB02/00234

(22) International Filing Date: 21 January 2002 (21.01.2002)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
0101513.0 19 January 2001 (19.01.2001) GB

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(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, OM, PH, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZM, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:
— *without international search report and to be republished upon receipt of that report*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 02/057301 A2

(54) Title: GENE SILENCING SUPPRESSOR

(57) Abstract: A plant virus protein, displaying PTGS suppressor activity, and the use thereof as a suppressor of the gene silencing mechanism in a non-plant host cell (i.e. an animal cell) is provided. Examples of the plant virus protein include cysteine-rich proteins, such as the 16K CRP of tobacco rattle virus or the 12K CRP of pea early browning virus. Also described is a method of suppressing or inhibiting a gene silencing mechanism in a non-plant orgnism or cell through the use of a plant virus protein, exhibiting post transcriptional gene silencing suppressing functions.

GENE SILENCING SUPPRESSOR

The present invention relates to the suppression of post-transcription gene silencing due to the presence of a cysteine-rich plant virus protein in the host.

Plants employ post transcriptional gene silencing (PTGS) as a mechanism to defend themselves against infection by viruses. In response, some plant viruses encode proteins that suppress PTGS and, thus, increase virus pathogenicity. Here we show that the *Tobacco rattle virus* (TRV) 16K cysteine-rich protein (CRP) is a silencing suppressor that is required for efficient virus multiplication, both in protoplasts and plants. The 16K protein can be substituted by the 12K CRP from the related virus, *Pea early-browning virus* and by the 2b protein, a known silencing suppressor, from the unrelated virus, *Cucumber mosaic virus*.

Expression of the 16K CRP and other PTGS suppressors in a *Drosophila* cell system prevents silencing of a

lacZ gene, demonstrating directly that the 16K CRP is a suppressor of post-transcriptional gene silencing. These results identify the tobravirus CRPs as a new class of PTGS-suppressors and suggest that the CRPs encoded by other, unrelated, plant viruses may also be silencing suppressors.

Plant viruses vary considerably in their ability to infect different plant species so that, for example, *Cucumber mosaic virus* (CMV) is known to infect more than 775 species from at least 85 different families (Palukaitis *et al.*, 1992), whereas, *Potato leafroll virus* infects only about 20 species, most of which are from the family Solanaceae (Harrison, 1984). A particular virus might be unable to infect a plant species because the plant lacks a protein that is required for efficient replication of the virus, for example, *Arabidopsis* TOM1 (Yamanaka *et al.*, 2000).

Alternatively the plant may actively resist infection by the virus, for example, tobacco plants carrying the N gene are resistant to strains of *Tobacco mosaic virus* (TMV) but are not resistant to other tobamoviruses (Whitham *et al.*, 1994). A more general resistance mechanism, referred to as systemic acquired resistance (SAR), also exists in tobacco and other plants (Sticher *et al.*, 1997). In this process, challenge to the lower leaves of a plant with TMV (or other unrelated pathogens) induces partial resistance to TMV (or other viruses) in upper, uninoculated leaves. SAR is dependent on the

synthesis of salicylic acid (SA) and exogenous application of SA can induce resistance to viruses. However, SA-mediated resistance appears to act by several mechanisms, as TMV is inhibited at the stage of virus replication, whereas CMV is blocked in systemic movement (Chivasa *et al.*, 1997; Naylor *et al.*, 1998).

More recently it has been discovered that plants also may employ a cytoplasmic, sequence-specific, RNA degradation system known as post transcriptional gene silencing (PTGS) to combat virus infection. PTGS in plants was first identified as the cause of cosuppression, in which transformation of plants with an additional copy of a host gene could abolish expression of both the transgene and host homologue by degradation of cytoplasmic mRNA (Napoli *et al.*, 1990). This system was shown to act on viruses, firstly by the demonstration that transformation of plants with non-translatable viral sequences often resulted in either extreme resistance or in a "recovery" phenotype where plants developed resistance following an initial, virus-susceptible phase (Lindbo *et al.*, 1993). In other studies it was shown that plants in which expression of a β-glucuronidase (GUS) transgene had been silenced by PTGS were protected against infection by a plant virus that carried part of the same (GUS) gene (English *et al.*, 1996). Furthermore, it was shown that host gene or transgene expression could be silenced following infection with a virus carrying

part (or all) of the same gene (Kumagai *et al.*, 1995; Ruiz *et al.*, 1998).

Several approaches have been taken to examine in detail the interaction of plant viruses with the gene silencing process. Infection with CMV of plants that carried post-transcriptionally silenced transgenes (GUS and nitrate reductase) led to the reversal of PTGS and resumption of transgene expression in newly emerging leaves (Beclin *et al.*, 1998). Earlier work had identified the CMV 2b protein as a pathogenicity determinant that is required for symptom formation and systemic invasion of particular hosts (Ding *et al.*, 1995). Silencing of a green fluorescent protein (GFP) transgene in *Nicotiana benthamiana* plants was reversed following infection with *Potato virus X* (PVX) that had been modified to express the CMV 2b gene, demonstrating that the 2b protein is a silencing suppressor (Brigneti *et al.*, 1998). This and other studies showed that the HC-Pro proteins of *Potato virus Y* and *Tobacco etch virus* can also function as silencing suppressors (Anandalakshmi *et al.*, 1998; Kasschau *et al.*, 1998). Evidence that PTGS is a defensive system that can target viruses comes from studies of Arabidopsis mutants (*sgs2* and *sgs3*) that originally were isolated in a screen for plants impaired in the silencing of a GUS transgene (Elmayan *et al.*, 1998). These plants have an increased susceptibility to CMV, showing that the PTGS system does provide some protection against this virus (Mourrain *et al.*, 2000). Interestingly, the

sgs mutants do not have increased susceptibility to two other viruses, *Turnip mosaic virus* and *Turnip vein clearing virus*. Plant virus-encoded silencing suppressors may target different components of the PTGS system. For example, the CMV 2b protein prevents initiation of silencing in newly emerging tissues but has no effect on already established silencing. In contrast the potyvirus HC-Pro protein suppresses silencing in all tissues (Brigneti *et al.*, 1998).

A survey of a small number of other plant viruses showed that the comovirus *Cowpea mosaic virus*, the geminivirus *African cassava mosaic virus*, the potexvirus *Narcissus mosaic virus*, the tobamovirus TMV, the sobemovirus *Rice yellow mottle virus* (RYMV), the tombusvirus *Tomato bushy stunt virus* (TBSV) and the tobravirus *Tobacco rattle virus* (TRV) also were able to suppress GFP silencing (Voinnet *et al.*, 1999). Although in this study the potexvirus PVX did not suppress silencing, using a different assay these authors showed that PVX is in fact able to prevent systemic silencing (Voinnet *et al.*, 2000). Thus, it seems probable that many plant viruses encode proteins that allow them to evade or inhibit PTGS in certain plant species, and that different suppressors target different parts of the PTGS pathway.

Inhibiting gene silencing in a host organism allows an increase and/or in certain cases renders possible the expression of a transgene which can be introduced

within the organism either through a foreign vector or through the genetic modification of the host organism itself.

Thus suppression or inhibition of the gene silencing mechanism may have many therapeutical implications, especially in gene therapy for humans, when a foreign gene expressing, for example, a therapeutic substance needs to be introduced and expressed in a host cell.

Statement of the invention

According to the present invention there is provided a method of suppressing or inhibiting gene silencing mechanisms in a non-plant host cell through the use of a plant virus protein exhibiting post-transcriptional gene silencing (PTGS) suppressing functions. Preferably the non-plant cells are animal cells or insect cells and more particularly may be mammalian cells.

The invention further provides a vector which comprises a polynucleotide sequence which encodes the above PTGS suppressor protein, and which advantageously expresses said polynucleotide sequence in a non-plant cell.

Typically, the PTGS suppressor protein is, or is derived from, the HC-Pro protein of *tobacco etch virus* (TEV), the 2b protein of *cucumber mosaic virus* ~~(CMV)~~, the ORF0 protein of *potato leafroll virus*

(PLRV), the 16K CRP protein of the *tobacco rattle virus*, the 12K CRP protein of *Pea early browning virus* and functional equivalents thereof. The term "functional equivalent" refers to modifications of the proteins, wherein the modifications do not adversely affect the ability of the protein (relative to the wild-type form) to suppress gene silencing. Also included are related proteins having, for instance 60% or more homology, preferably 75% or more homology, especially 80% or 90% or more homology with the protein in question. Desirably the related protein will show 95% or more (even 98% or 99%) homology with the protein in question. Amongst functional equivalents of the PTGS suppressor protein, HC-Pro, 2b, ORF0 and 16K CRP or corresponding proteins of other related plant viruses are particularly preferred.

A further object of the invention is a method to increase the expression and/or replication of a virus in a host cell, said method comprising reduction of post-transcriptional gene silencing by said host cell by expression of a plant virus protein in said non-plant host cell.

Another object of the invention is a method for protecting a heterologous viral vector or transgene from post-transcriptional gene silencing in a non-plant host cell, the heterologous viral vector or transgene expressing at least one heterologous protein of interest in the host cell, said method

comprising the expression of a plant virus protein in said host cell.

A further object of the invention is a method to increase the yield of expression of a heterologous protein expressed by a viral vector or transgene in a non-plant host cell, said method comprising the expression of a plant virus protein in said host cell.

The method of the present invention finds particular utility in the production of a protein of interest (for example a therapeutic protein) in a biofactory, such as an animal organism.

Any heterologous protein (which term is deemed to include small protein molecules or peptides) may be used in the present invention and there is no particular limitation as to size (which in any event would depend only upon the viral vector, if used). Mention may be made of antibodies (including antibody-type molecules such as ScFv, for example), protein hormones such as GnRH, insulin or the like, cellular receptors or other biologically active proteins as being exemplary of heterologous proteins of interest. The heterologous protein will in general be a pre-determined molecule which is specifically selected for expression.

The invention further relates to a vector which comprises a ~~polynucleotide~~ sequence which encodes the

above PTGS suppressor protein, and which expresses said polynucleotide sequence in the non-plant host cell.

The reference to "plant virus protein" refers not only to the full-length wild type version of the protein, but also to variations of such proteins to include minor modifications thereto (for example amino acid deletions, insertions or substitutions) which do not adversely affect its ability to protect heterologous vectors or transgenes from gene silencing by the host cell.

More particularly in one embodiment the plant virus protein is a cysteine-rich protein. Examples of such cysteine-rich protein include the 16K CRP protein, in particular the 16K CRP protein of the *tobacco rattle virus*, or its functional equivalent. In another embodiment the cysteine-rich protein is the 12K CRP of *Pea early browning virus* or its functional equivalent.

Whilst protection of nucleic acid, and particularly RNA, from silencing machinery is exemplified in plant cells and insect cells, protection in other types of host organisms, such as animals, fungi etc. using the cysteine-rich plant virus protein expressed transgenically, from viral vectors or by other ways, in medical therapy/gene, therapy/over-expression systems, for example in yeast/fungi (fermentation) falls within the scope of the present invention.

Although the previous study showed that TRV was able to suppress transgene silencing, the specific viral protein responsible for this activity was not identified. TRV, like the other tobraviruses *Pea early-browning virus* (PEBV) and *Pepper ringspot virus*, has a bipartite, positive strand RNA genome (MacFarlane, 1999). The larger RNA (RNA1) encodes the 134K and 194K proteins that comprise the viral replicase, a 29K cell-to-cell movement protein, and a 16K cysteine-rich protein (CRP). The smaller RNA (RNA2) varies considerably between isolates, but always encodes the coat protein (CP) and may encode other (2b and 2c) proteins involved in virus transmission by nematodes. A characteristic of the tobraviruses is that RNA1 can infect plants systemically in the absence of RNA2; i.e. without CP expression and virion formation. This type of infection, referred to as NM-infection, occurs frequently in vegetatively propagated crop plants such as potato and bulbous ornamentals, and is often associated with increased symptom severity. Clearly, therefore, RNA1 encodes all the functions necessary for virus multiplication including, possibly, suppression of PTGS/host defence. As the TRV 16K protein is the one protein encoded by RNA1 without an assigned function we investigated whether it played a role in viral replication and pathogenicity.

We have found that the 16K CRP acts to suppress gene silencing in plant and animal cells.

Thus the invention also relates to the use of a cysteine-rich plant virus protein displaying PTGS suppressor activity as a suppressor of the gene silencing mechanism of a host cell.
Preferred host cells are plant cells, for example tobacco plants. Whilst the present invention will normally contemplate the use of whole plants or plantlets as the host cells, limited infection of certain parts of the plant may also be utilised, as of course may be the use of protoplasts or other *in vitro* cell cultures. Other host cells include insect cells, especially cell cultures thereof.

The present invention will now be further described with reference to the following, non-limiting, examples and figures in which:

Figure Legends

Figure 1. Deletion of the 16K gene prevents virus infection. **(A)** Northern blot of RNA extracted from *N. tabacum* leaves 7 days post inoculation (dpi). Lanes 1-6, inoculated with wild type RNA1 (transcript from pTRV1) and RNA2-GFP (transcript from pK20-GFPc). Lanes 7-12, inoculated with transcripts of pTRV1-16ßand pK20-GFPc. M is RNA from uninoculated plants. C is RNA of *N. benthamiana* infected with wild type TRV. Blot hybridised with probes specific for TRV RNA1 and RNA2. Location of RNAs 1 and 2 is indicated. **(B)** GFP expression, viewed under UV light,

in tobacco leaves inoculated with transcripts of pK20-GFPc and pTRV1 (bottom, left), pTRV1NB (top, left), pTRV1-12 (top, right) or pTRV1-16Δ (bottom, right).

Figure 2. Comparison of 16K and 12K CRPs. **(A)** Alignment of amino acid sequences of tobravirus CRPs generated by the Clustal W programme. Asterisks identify residues that are identical between the 16K and 12K proteins. Hyphens indicate spaces inserted to maximise alignment. Residues forming the CRP motif identified by Diao *et al.*, 1999 are in solid boxes. C-terminal basic domain is boxed in dashed lines. **(B)** Schematic drawing of constructs used in this work. Asterisk in pTRV1 represents the leaky terminator present in the TRV replicase protein. 1a/MP denotes the cell-to-cell-movement protein. The single and double terminators inserted into the 16K genes of pTRV1-16stop and pTRV1-16dstop appear as asterisks below the 16K gene.

Figure 3. Multiplication of 16K mutants. **(A)** Northern blot of RNA extracted from *N. benthamiana* plants at 6dpi (inoculated leaf) and 11dpi (systemic leaf). Lanes 1-3, wild type RNA1 (pTRV1) and RNA2-GFP (pK20-GFPc). Lanes 4-6, pTRV1NB and pK20-GFPc. Lanes 7-9, pTRV1-16Δ and pK20-GFPc. Lanes 10-12, pTRV1-12 and pK20-GFPc. M is RNA from an uninoculated plant. Blot hybridised with probes specific for TRV RNA1 and RNA2. **(B)** GFP-fluorescence indicating systemic movement of TRV derived from pTRV1-12 and

pK20-GFPc. **(C)** Northern blot of RNA extracted from *N. benthamiana* protoplasts 48 hours after inoculation with transcripts of wild type RNA2 (pK20-RNA2) and (1) pTRV1, (2) pTRV1NB, (3) pTRV1-16β, (4) pTRV1-12. M is from protoplasts electroporated without transcript. C is RNA from plants infected with TRV. Blot hybridised with probes specific for TRV RNA1 and RNA2. RNA1 can be seen in lanes 3 and 4 after very long exposure of the blot, and in other protoplast experiments. rRNA denotes ribosomal RNAs in these samples, labelled by ethidium bromide staining. **(D)** Northern blot of RNA extracted from leaves of *N. benthamiana* inoculated with transcript RNA2 from pK20-GFPc and transcript RNA1 from pTRV1NB (lanes 1 and 2) or pTRV1-16stop (lanes 3-7). Blot hybridised with probes specific for TRV RNA1 and RNA2.

Figure 4. The 16K gene is a pathogenicity determinant. **(A)** Symptoms on *N. tabacum* var. *Samsun* NN following infection by TRV RNA1 (top, left), TRV RNA1 and RNA2 (top, right) or TRV RNA1 and RNA2-16K. Expression of an additional copy of the 16K gene from RNA2 (RNA2-16K) results in severe stunting and necrosis of the plants. **(B)** Northern blot of RNA extracted from systemically-infected leaves of plants photographed in (A). Lanes 1-3, RNA1-only infection. Lanes 4-6, infection with RNA1 and RNA2. Lanes 7-9, infection with RNA1 and RNA2-16K. Position of viral RNAs is indicated by arrows. 1 is RNA1, 2a is wild type RNA2, 2b is RNA2-16K. rRNA denotes ribosomal RNAs in these samples, labelled by ethidium bromide

staining. Blot hybridised with probes specific for TRV RNA1 and RNA2.

Figure 5. Heterologous expression of the 16K gene. **(A)** Upper, uninoculated leaves of tobacco inoculated with transcripts of PVX (left) and PVX-16K (right). **(B)** *N. benthamiana* plants 20 days after inoculation with transcripts of PVX (left) and PVX-16K (right). The plants infected with PVX continued to grow after this time, whereas, the severe tip necrosis of the plants infected with PVX-16K was fatal.

Figure 6. Complementation of a 16K mutation *in trans*. **(A)** Northern blot of RNA samples of *N. benthamiana* inoculated with transcripts of (lanes 1-4) pTRV1NB and pK20-GFPc, (lanes 5-8) pTRV1NB and pK20-16K, (lanes 9-12) pTRV1-16dstop and pK20-GFPc, (lanes 13-16) pTRV1-16dstop and pK20-16K. Expression of the 16K gene from RNA2 complements the early termination mutations in the 16K gene on RNA1. M is RNA from uninoculated plants. Blot hybridised with probes specific for TRV RNA1 and RNA2. The position of viral RNA 1 and 2 indicated by arrows. **(B)** Northern blot of RNA samples of *N. benthamiana* inoculated with transcripts of (lanes 1-4) pTRV1-16dstop and pK20-GFPc, (lanes 5-8) pTRV1-16dstop and pK20-CMV2b, (lanes 9-12) pTRV1-16dstop and pK20-16K. Expression of the CMV 2b gene from RNA2 complements the early termination mutations in the 16K gene on RNA1. Blot hybridised with probes specific for TRV RNA1 and RNA2.

Figure 7. Suppression of gene silencing in *Drosophila* cells by the TRV 16K protein.
(A) Representative field of view of cells transfected with a plasmid expressing the *lacZ* gene alone, or **(B)** with this plasmid and dsRNA to induce silencing, or **(C)** with this plasmid, dsRNA and a second plasmid expressing the 16K protein.
(D) The percentage of cells in a culture expressing the *lacZ* gene in a transient assay.
Cells were transfected with a plasmid expressing the *lacZ* gene alone (*lacZ*); or with the plasmid and dsRNA to induce silencing (*lacZ* + dsRNA); or with the plasmid, dsRNA and a second plasmid expressing the 16K protein (*lacZ* + dsRNA + 16K). A minimum of 100 cells in each of 5 representative fields of view (i.e. >500 cells) was counted and the percentage staining positive for *lacZ* expression was determined.

EXAMPLE 1: TRV 16K CRP IN PLANTS

Materials and methods

Construction of full-length clone of TRV RNA1

Single-stranded cDNA was synthesised from total RNA extracted from *Nicotiana benthamiana* plants infected with TRV isolate PpK20 as described previously (MacFarlane *et al.*, 1991). A full-length clone of RNA1 was amplified using a proof-reading polymerase with primers designed to include a T7 RNA polymerase promoter sequence and *SmaI* restriction site at the 5'

and 3' ends, respectively, of the virus sequence, and ligated into plasmid pCR-TOPO-XL according to the manufacturer's instructions (Invitrogen). The full-length clone, pTRV1, was linearised with *Sma*I, and transcribed using T7 RNA polymerase (Ambion Inc.). Transcripts were capped by addition of diguanosine triphosphate to the transcription reaction (MacFarlane *et al.*, 1991).

Mutation of the 16K gene to produce pTRV1NB, pTRV1-16Δ, pTRV1-12 and pTRV1-16stop

Inverse PCR was used to introduce a *Nde*I site immediately upstream of the 16K initiation codon and a *Bgl*II site immediately after the 16K termination codon. A fragment carrying these mutations was moved into the full-length cDNA clone to produce plasmid pTRV1NB. Subsequently, the 16K gene was deleted by digestion with *Nde*I and *Bgl*II, blunting with Klenow polymerase and religation to produce plasmid pTRV1-16Δ.

The 12K gene from RNA1 of PEBV isolate SP5 was PCR amplified to include an upstream *Nde*I site and a downstream *Bgl*II site. This fragment was inserted into pTRV1NB in place of the 16K gene to produce plasmid pTRV1-12.

The NdeI-BglII fragment carrying the 16K gene was reamplified using a mutagenic primer (TCCATATGACGTGTGTACTC**TAG**GGTTGTGTGAATGAAGTCACTGTT)

(SEQ ID No: 1) to introduce an early terminator (bold) at position 6126, 16 nucleotides downstream of the 16K initiation codon (underlined). The fragment was moved into the full-length clone pTRV1NB to produce plasmid pTRV1-16stop.

Inverse PCR was used to introduce two early terminators at positions 6120 and 6126 (bold), 10 and 16 nucleotides downstream of the 16K initiation codon (underlined) to produce the sequence ATGACGTGT**TAA**CTC**TAG**...(SEQ ID No: 2) A fragment incorporating these changes but lacking the *Nde*I and *Bgl*II 16K-flanking sites was moved into the full-length clone pTRV1 to produce plasmid pTRV1-16dstop.

Expression of heterologous viral genes from TRV RNA2

The CMV 2b gene was amplified from a full-length cDNA clone of RNA2 of CMV isolate Fny to incorporate *Nco*I and *Kpn*I sites at the 5' and 3' ends of the gene, respectively. The *Nco*I-*Kpn*I fragment was used to replace the GFP gene carried on a similar fragment in the TRV virus vector plasmid pK20-GFPc (MacFarlane and Popovich, 2000). This new construct, pK20-CMV2b, expresses the CMV 2b protein from a duplicated tobravirus CP subgenomic promoter in TRV RNA2. Similar strategies were used to clone the PEBV 12K gene, as a *Rca*I-*Eco*RI fragment, and the TRV 16K gene, as a *Rca*I-*Kpn*I fragment, into TRV RNA2 to produce, respectively, plasmids pK20-12K and pK20-16K.

Inoculation and analysis of plants

Leaves of small *N. benthamiana* or *N. tabacum* cv. Samsun NN plants were dusted with carborundum and mechanically inoculated with capped transcripts of TRV RNA1 and RNA2. RNA was isolated from samples of inoculated and systemically infected leaves at 5-7 dpi and 10-12 dpi, respectively, and analysed by northern blotting as described before (MacFarlane *et al.*, 1991) except that complementary-strand, RNA probes were prepared using a non-radioactive system (AlkPhos, Amersham Pharmacia).

Protoplasts were isolated from *N. benthamiana* plants as described before (Power and Chapman, 1985) and inoculated with transcript RNA by electroporation. RNA was extracted after 48 hours and analysed by northern blotting.

Results

The 16K gene is required for virus multiplication

The initial step in this work was the construction of a full-length cDNA clone of RNA1 of TRV isolate PpK20. Transcripts derived from this clone, pTRV1, were infectious when inoculated to plants either alone or in combination with transcripts of TRV PpK20 RNA2 (Mueller *et al.*, 1997). Unlike the previously described clone of TRV RNA1 (Hamilton and Baulcombe, 1989), transcripts from pTRV1 were encapsidated into

virus particles, and could be transmitted by the natural nematode vector of TRV (data not shown). A modified clone of TRV RNA1 was created in which the 16K gene was flanked by novel restriction sites.

Transcripts from this clone, pTRV1NB, behaved in an identical way to those derived from the wild type clone pTRV1. A second clone, pTRV1-16Δ, was created in which the entire 16K gene was deleted. Transcripts derived from clones pTRV1NB or pTRV1-16Δ were mixed with RNA2 transcripts from clone pK20-GFPc and inoculated to *Nicotiana tabacum* (var. Samsun NN). Fluorescent lesions were visible by three days post inoculation (dpi) on four of six plants inoculated with wild type (pTRV1NB) RNA1 and RNA2-GFP, however, no fluorescent lesions were visible on any plant inoculated with RNA1 carrying the 16K gene deletion even at 6dpi or later. Northern blot analysis showed that, although both TRV RNAs were clearly evident in plants inoculated with wild type transcripts, neither RNA1 nor RNA2 could be detected in plants inoculated with transcripts from the 16K deletion mutant (Fig. 1A).

The 3' proximal open reading frame of RNA1 of all three tobraviruses encodes a small, cysteine-rich protein (CRP). The CRP from PEBV is smaller (12K) than the TRV 16K protein, however, both proteins contain cysteine/histidine motifs reminiscent of zinc-binding domains present in some regulatory proteins and both have C-terminal regions rich in

basic amino acid residues (Fig. 2A). Amino acid sequence identity between the 16K and 12K proteins is low (31%), however, there is a striking conservation in the arrangement of the cysteines and their flanking residues in these proteins. Thus, clone pTRV1-12 was constructed to determine whether the similarities in the cysteine-rich domains would enable the PEBV 12K protein to function in place of the TRV 16K protein (Fig. 2B). Inoculation of *N. tabacum* with transcripts of pTRV1-12 and pK20-GFPc produced isolated, very small fluorescent lesions on only two of five plants at 5 dpi, whereas, in the same experiment all five plants inoculated with wild type transcripts carried many, large fluorescent lesions by this time (Fig. 1B). As before, transcripts from pTRV1-16Δ were apparently not infectious.

Inoculation of *N. benthamiana* with transcripts from these clones gave slightly different results. Although mutant TRV1-12 was not as infectious as wild type TRV1 or TRV1NB, viral RNAs were clearly detectable in both inoculated and systemically infected leaves (Fig. 3A) and GFP fluorescence was apparent in systemic leaves (Fig. 3B). Viral RNAs were barely detectable in leaves inoculated with mutant TRV1-16Δ, and were not detected in upper, uninoculated leaves samples at 11dpi. Lack of systemic movement of mutant TRV-16Δ was confirmed by RT-PCR analysis of these samples (data not shown). In

N. benthamiana protoplasts, mutants TRV1-16Δ and TRV1-12 both accumulated to much lower levels than did the wild type viruses, suggesting that the 16K gene is required for efficient replication of TRV and that, in these conditions, the PEBV 12K gene is not an adequate replacement (Fig. 3C).

The requirement of the 16K protein for efficient TRV replication was further examined by the creation of two mutants carrying premature translation termination codons in the 16K gene. In mutant TRV-16stop, the sixth codon of the 16K gene is replaced by a UAG terminator and the 16K gene is flanked by *Nde*I and *Bgl*II sites. This mutant multiplied very poorly compared to wild type virus both in whole plants (Fig. 3D) and in protoplasts (data not shown), confirming that the 16K protein rather than the 16K RNA sequence is required for efficient virus replication. In mutant TRV-16dstop, the fourth codon is UAA and the sixth codon is UAG, however, the 16K gene is not flanked by artificial *Nde*I and *Bgl*II restriction sites. This mutant also replicated poorly confirming that the non-viral restriction sites introduced into all of the previous mutants were not the cause of reduced replication efficiency.

The 16K protein is a pathogenicity determinant

TRV RNA2 can be used as a vector from which heterologous sequences are expressed at high levels using a duplicated coat protein promoter (MacFarlane

and Popovich, 2000). Clone pK20-16K was constructed to examine the effects of over-expression of the 16K protein on symptom production by TRV. Infection of tobacco plants with TRV RNA1 only (NM infection) resulted in stem/mid-vein necrosis and slight stunting but on most systemic leaf blades no symptom formed. Infection with wild type (RNA1 and RNA2) TRV (isolate PpK20) resulted in infrequent, small necrotic patches on systemic leaves together with some leaf distortion and chlorotic mottle. Infection with TRV RNA1 and RNA2-16K caused severe stunting and distortion of systemic leaves together with widespread necrosis (Fig. 4A). Northern blot analysis of these plants showed that NM-infected plants had little or no virus RNA in systemic leaf blades. Both viral RNAs were easily detectable in wild type virus-infected tissue. However, infection with TRV RNA1 and RNA2-16K led to an increase in the level of virus RNAs, particularly RNA2 (Fig. 4B). Thus, over-expression of the 16K protein leads to increased pathogenicity of TRV.

Expression of the 16K gene from a PVX vector

To examine whether enhancement of symptom expression by the 16K protein was specific for TRV, the 16K gene was cloned into the PVX vector (Chapman *et al.*, 1992). Inoculation of tobacco plants with PVX lacking any insert resulted in a systemic, chlorotic mottle (Fig. 5A, left). Systemic infection with PVX-16K was slower by 1 to 2 days than with PVX and

produced severe, chlorotic lesions rather than mottling (Fig. 5B, right). There was an even greater contrast in symptomatology of the two viruses following inoculation to *N. benthamiana*. Both PVX and PVX-16K initially induced severe systemic leaf curling and vein chlorosis. However, by 20dpi, whereas PVX-infected plants were highly stunted, plants infected with PVX-16K were killed (Fig. 5B). Thus, the TRV 16K protein is a pathogenicity determinant that can function when expressed from a different virus.

Complementation of 16K mutation by the gene encoding the CMV 2b silencing suppressor

As expression of the 16K gene from TRV RNA2 enhanced the replication (and symptom production) of wild type TRV RNA1, experiments were carried out to test the effect of this RNA2 on the replication of the 16dstop RNA1 mutant. Virus could not be detected by northern blotting of plants inoculated with 16dstop RNA1 and RNA2-GFP, either in inoculated or systemic leaves (Fig. 6A, lanes 9-12). However, inoculation with 16dstop RNA1 and RNA2-16K produced a readily detectable infection (Fig. 6A, lanes 13-16). Thus, expression of the 16K protein *in trans* completely rescued the very poorly replicating 16dstop RNA1 mutant. Likewise, other experiments showed that expression of the PEBV 12K CRP from TRV RNA2 was able to complement the 16dstop mutation (data not shown).

The results from experiments described above showed that the TRV 16K protein is a pathogenicity determinant that is required for efficient viral replication and, thereafter, systemic infection of plants. These properties are consistent with the 16K protein acting as a PTGS suppressor. We hypothesized that the absence of suppressor function resulting from the 16dstop RNA1 mutation could be overcome by co-expression of a host defense suppressor protein derived from another virus. Thus, 16dstop RNA1 was inoculated to plants together with transcripts of pK20-CMV2b, in which the CMV2b gene is expressed from TRV RNA2. Northern blotting showed that, indeed, the CMV 2b gene was able to rescue TRV carrying a mutation in the 16K gene, resulting in high levels of viral RNAs both in inoculated and systemic infected leaves (Fig. 6B, lanes 5-8). RT-PCR and sequencing confirmed that the 16dstop mutation was retained in RNA1 and that the CMV 2b gene was retained in RNA2

Discussion

In this study, we examined the role of the 16K protein in the replication and pathogenesis of TRV. An earlier report suggested that the 16K gene was dispensable for TRV multiplication (Guilford *et al.*, 1991). Our results conflict with those of this previous study, as we show that mutation of the 16K gene leads to a significant decrease in the accumulation of virus RNA in infected plants. Protoplast studies confirmed that the 16K protein is

required for efficient virus replication, and over-expression of the 16K protein, whether from TRV or from PVX, led to an increase in the severity of symptoms. Expression of the 16K protein from TRV RNA2 functions *in trans* to complement a mutation in the RNA1-encoded 16K gene. Also, mutation of the 16K gene was overcome by incorporation of the gene encoding the CMV 2b silencing suppressor protein into TRV RNA2, suggesting that the 16K protein itself might be a silencing suppressor. We have used a novel, insect cell expression system to confirm that the TRV 16K protein is a suppressor of gene silencing which may explain how alteration in the level of 16K expression has such a significant effect on virus pathogenicity.

Mutation of some of the other virus genes recently identified as encoding silencing suppressors results in a wide range of effects. The CMV 2b protein was shown not to be required for systemic infection of *N. glutinosa*, although RNAs 1 and 2 accumulated to lower levels and symptoms produced by a 2b mutant were much reduced and delayed in appearance compared to those of the wild type virus (Ding *et al.*, 1995). In inoculated leaves of cucumber, the 2b mutant accumulated to much lower levels (<5%) than did the wild type virus, and did not move systemically. Whether these phenotypes resulted from reduction in virus replication or from specific failures in virus movement is not known. The potyvirus HC-Pro protein is multifunctional, being involved in virus

transmission by aphids, autoproteolytic cleavage between itself and the downstream P3 protein, genome amplification and long-distance virus movement (Govier *et al.*, 1977; Carrington *et al.*, 1989; Cronin *et al.*, 1995; Kasschau *et al.*, 1997). A number of insertion mutations that were introduced into the *Tobacco etch virus* (TEV) HC-Pro gene did not affect autoproteolytic function but did suppress virus replication in protoplasts (Cronin *et al.*, 1995). One mutant (IGN) which accumulated to levels less than 1% of the wild type virus was, however, able to move systemically and induce mild, systemic symptoms. In contrast, another mutant (CCCE) accumulated in protoplasts to 25% of the level of wild type virus but was incapable of moving into upper leaves. These results suggested that HC-Pro has separate functions associated with virus replication and movement. The TBSV silencing suppressor has been identified as the p19 protein that is nested within the p22 cell-to-cell movement protein gene near the 3' terminus of the virus RNA (Voinnet *et al.*, 1999). Expression of the p19 protein is required for systemic spread of the virus in spinach, induction of a hypersensitive response in *N. tabacum* and induction of systemic lethal collapse in *N. benthamiana* (Scholthof *et al.*, 1995a; 1995b). However, in *N. benthamiana* protoplasts mutation of the p19 gene had no effect on virus replication (Chu *et al.*, 2000). Perhaps the results obtained following mutation of the gene encoding the RYMV P1 silencing suppressor are most similar to our findings with the TRV 16K gene. The

RYMV P1 protein is encoded by the 5' terminal open reading frame of the viral RNA. Deletion of the entire gene or insertion of a premature termination codon into the gene abolished replication of viral RNA in protoplasts. A mutant in which the P1 initiation codon was removed was able to replicate at reduced levels (c. 50% of wild type) in protoplast but did not accumulate either in inoculated, or in upper uninoculated leaves of whole plants (Bonneau *et al.*, 1998). As with some of the silencing suppressors discussed above, it is possible that in some plant species, mutation of the TRV 16K gene may not be deleterious. Deletion or frameshift mutation of the PEBV 12K gene produced similar results in *Nicotiana* species to those obtained here for the TRV 16K gene, with a c. 60-fold reduction in accumulation of virus RNAs (S. MacFarlane, unpublished). In contrast, in pea the PEBV 16K deletion mutant accumulated to wild type levels, although the 16K frameshift mutant could not be detected (Wang *et al.*, 1997).

A premature termination mutation of the 16K gene was overcome by co-expression of the CMV 2b gene from TRV RNA2. The 2b protein is known to intervene at the stage of PTGS initiation (Brigneti *et al.*, 1998) and could, thus, be able to prevent a silencing-based defence reaction being initiated against the TRV 16K mutant. As yet, there are no data to explain whether the TRV 16K protein acts against PTGS initiation, or during the later maintenance phase (as is the case

for the potyvirus HC-Pro protein). There are a few other examples where the pathogenicity of one virus has been modified by co-expression of a silencing suppressor derived from a different virus. Expression of the potyvirus HC-Pro protein in transgenic plants showed it to be the determinant that mediates increases in PVX multiplication and pathogenicity during PVX/potyvirus synergism (Vance *et al.*, 1995). When the 16K gene was expressed from the PVX vector, there was an increase in the severity of disease symptoms in a similar way to when other silencing suppressors were expressed from PVX (Scholthof, *et al.*, 1995b; Brigneti, *et al.*, 1998; Voinnet, *et al.*, 1999; Lucy *et al.*, 2000). Only one other example has been reported in which a silencing suppressor from one virus has been completely replaced with that of another virus (Ding *et al.*, 1996). In this experiment the 2b gene of CMV was replaced with the homologous gene from another cucumovirus *Tomato aspermy virus* (TAV). Unexpectedly, the hybrid virus had a significantly increased pathogenicity compared to either of the parental viruses (Ding *et al.*, 1996). TRV and CMV are taxonomically very distinct, and there is no significant amino acid sequence similarity between the 16K and 2b proteins. Nevertheless, these proteins are functionally equivalent in the protection of TRV against host defence mechanisms.

The TRV 16K protein was detected by western blotting in extracts of infected tobacco protoplasts (Angenent

et al., 1989). The 16K protein accumulated to high levels, equivalent to that of the coat protein (CP), but continued to be expressed even after CP synthesis had declined. Cell fractionation experiments, combined with sedimentation analysis, showed that the 16K protein accumulated in a high-molecular weight complex, either as a multimer or in association with host proteins (Angenent *et al.*, 1989). It is tempting to speculate that the 16K protein may associate with proteins of the host silencing system, thus, inhibiting their action against TRV. In whole plants the 16K protein was only detected when infected leaves were extracted using highly denaturing reagents, although, even in these conditions some of the protein still accumulated in higher molecular weight aggregations (Liu *et al.*, 1991). Immunogold labelling of ultrathin sections showed that the 16K protein was located both in the cytoplasm but mostly in the nucleus (Liu *et al.*, 1991). Interestingly, the CMV 2b protein also localises to the nucleus, and removal of an arginine-rich domain at the N-terminus of the protein abolished both transport into the nucleus and silencing suppressor activity (Lucy *et al.*, 2000). The TRV 16K and PEBV 12K proteins also possess an arginine-rich domain, at the C-terminus of the proteins, which might function as a nuclear localisation signal (Fig. 2A). Computer alignment suggested that there might be significant amino acid sequence homology between the C-terminal basic domain of the TRV 16K protein and mammalian high mobility

group chromatin (HMG) proteins (Koonin *et al.*, 1991). HMG proteins are nuclear proteins that bind DNA in a non-sequence-specific fashion to promote chromatin function and gene regulation (Grasser, 1998).

Viruses belonging to the genera *Tobravirus* (TRV, PEBV), *Hordeivirus* (e.g. *Barley stripe mosaic virus*, BSMV), *Carlavirus* (e.g. Potato virus M), *Pecluvirus* (e.g. *Peanut clump virus*, PCV), *Furovirus* (e.g. *Soil-borne wheat mosaic* virus) and *Benyvirus* (*Beet necrotic yellow vein virus*, BNYVV) all encode a small (<20 kDa molecular weight) protein with an N-terminal or central cysteine-rich domain. Amino acid sequence alignment studies suggested that the tobravirus, pecluvirus, hordeivirus and furovirus proteins, in particular, share a region of seven cysteines, with a highly conserved central motif of Cys-Gly…Cys-Gly-X-X-His (Diao *et al.*, 1999). BSMV is the only one of these viruses for which a detailed study of the function of the CRP has been carried out. Virus in which the gene encoding the BSMV γb CRP had been deleted was able to infect barley plants systemically but virus RNAs accumulated to only 10-20% of wild type levels and virus CP expression was reduced by three orders of magnitude (Petty *et al.*, 1990). Site directed mutation of each of the individual cysteine and histidine residues identified above as part of the conserved CRP motif, caused the same phenotype as the complete deletion mutation, emphasising the importance of these residues in CRP function (Donald and Jackson, 1994). Other properties associated with

the BSMV γb protein are seed transmission of the virus (Edwards, 1995) and RNA-binding (Donald and Jackson, 1996). Similarly, the PEBV 12K CRP also is involved in seed transmission (Wang *et al.*, 1997) and can bind RNA (D. Wang and J. Davies, personal communication). The roles of the CRPs in furovirus and carlavirus biology are not known. However, mutation of the BNYVV P14 CRP greatly reduced the accumulation of virus RNA and had the additional effect of decreasing expression of CP (Hehn *et al.*, 1995). Also, frameshift mutation of the P15 CRP of PCV had a severe effect, reducing replication of the virus in protoplasts to very low levels (Herzog *et al.*, 1998). We suggest that, based on our findings on the function of the tobravirus 16K and 12K proteins, the CRPs from this diverse group of viruses may all act as suppressors of the plant PTGS system.

EXAMPLE 2: *Drosophila* cell gene silencing assay

Suppression of PTGS by the 16K protein

A system for studying gene silencing in cultured *Drosophila* cells was described recently in which transient expression of a *lac*Z gene can be prevented by co-transfection of the cells with double-stranded *lac*Z-specific RNA (Hammond *et al.*, 2000). We have shown that induction of *lac*Z silencing can be prevented by simultaneous expression of certain plant virus genes demonstrating that some plant viral silencing suppressors function in this heterologous

system (B. Reavy and S.A. MacFarlane, submitted). Expression of the TRV 16K protein in *Drosophila* cells also was found to prevent dsRNA-mediated silencing of *lacZ*, confirming our hypothesis that it is a silencing suppressor protein. When cells were transfected only with a plasmid (pMT/V5-His/lacZ) encoding the *lacZ* gene, ~ 50% of the cells stained blue after 48hr indicating the production of β-galactosidase. Co-transfection of cells with pMT/V5-His/lacZ and dsRNA corresponding to ~500nts at the 5' end of the *lacZ* gene resulted in only ~6% of cells staining blue, indicating that efficient silencing of lacZ had occurred. However, co-transfection of pMT/V5-His/lacZ with *lacZ*-specific, dsRNA and a plasmid carrying the 16K gene increased the number of cells staining blue to ~ 26%, demonstrating that the 16K protein inhibits RNA-mediated gene silencing (Fig. 7).

Results and Discussion

Analysis of gene silencing in *Drosophila* S2 cells was performed by transient expression using a variation of the assay described by Hammond *et al*. (2000). DS2 cells were transfected with a plasmid expressing β-galactosidase along with dsRNA corresponding to approximately the first 500 nts of the *lacZ* gene to induce silencing. Co-transfection of these two molecules along with a second plasmid expressing the TRV 16K protein was used to assay suppression of gene silencing.

Post-transcriptional gene silencing (PTGS), also known as RNA interference or RNA silencing, has been observed in a variety of organisms including plants, fungus (Ding, 2000), etc. The silencing involves sequence-specific degradation of a target RNA molecule and can be initiated by dsRNA homologous to the target RNA. PTGS has been used to generate resistance to viruses in transgenic plants (Waterhouse et al., 1998) but also appears to be an inherent virus resistance mechanism in plants (Covey et al, 1997; Ratcliff et al 1997; Elmayan et al., 1998; Ratcliff et al., 1999 Mourrain et al., 2000). A number of plant viruses have proteins that act as suppressors of PTGS and these can act at different stages of the suppression mechanism (Anandalakshmi et al, 1998; Brigneti et al, 1998; Kasschau & Carrington 1998; Voinnet et al, 1999; Lucy et al, 2000; Llave et al, 2000). PTGS has recently been demonstrated in cultured *Drosophila* cells and a sequence-specific nuclease involved in the process partially purified (Hammond et al, 2000). Here we show that a plant virus protein previously described as a suppressor of gene silencing also suppress gene silencing in *Drosophila* cells and also detect gene silencing suppression with a second plant virus protein. The HCPRO protein of tobacco etch virus (TEV) is able to reverse gene silencing in plants after it has been established and appears to affect a step involved in maintenance of PTGS (Anandalakshmi et al, 1998; Llave at al, 2000). Transient expression was used to determine if expression of this protein had any

suppressive effect on gene silencing in *Drosophila* cells. β-galactosidase activity could be detected by staining in up to approximately 70% of *Drosophila* cells when they were transfected with a *lacZ* expression plasmid alone (Fig 8A). The number of cells staining for β-galactosidase was only approximately 12% when dsRNA corresponding to approximately the first 500nts of the lacZ gene was co-transfected with the *lacZ* expression vector (Fig 8B). Co-transfection of *Drosophila* cells with dsRNA and *lacZ* and an HCPRO expression vectors resulted in staining of approximately 50% of the cells in the culture (Fig 8C). The number of cells staining when transfected with the *lacZ* expression vector alone varied somewhat between experiments presumably due to variation in the quality of plasmid DNA and the condition of the cells but there was little variation between replicate plates within an experiment.

The percentage of cells that stained for β-galactosidase when transfected with the *lacZ* expression vector alone was normalised to 100 and the ratio of the number of cells staining with the other treatments was expressed as a percentage of this for quantitation purposes in Fig 1E. The TEV HCPRO protein was effective in suppressing gene silencing in transient assays.

A stable cell line (DS2-HCPRO) expressing the HC-Pro protein was produced to attempt to improve the efficiency of the suppression assay by reducing the

number of co-transfected nucleic acid molecules from 3 to 2. An unrelated cell line (DS2-VCL) expressing a recombinant antibody was used as a control for silencing in order to eliminate the possibility that stable transformation of the cells could interfere with silencing. Co-transfection the *lacZ* expression vector and dsRNA produced a slight reduction in the number of DS2-HCPRO cells cells staining for β-galactosidase activity compared to the *lacZ* expression vector alone. A significantly greater silencing effect was seen in the DS2-VCL cells (Fig 9). Quantitation of the numbers of cells staining showed that significantly more cells stained for β-galactosidase activity after transfection with the *lacZ* expression vector and dsRNA in the DS2-HCPRO cells than in the DS2-VCL cells (Fig 10). The numbers of cells that stained for β-galactosidase activity in the DS2-HCPRO cells in the presence of the *lacZ* expression vector and dsRNA was slightly higher than when DS2 cells were transiently transfected with pMT/V5-His/lacZ along with pMT-HCPRO and lacZ dsRNA indicating that suppression of silencing was somewhat more efficient in the DS2-HCPRO cells.

Transient transfection with mutant HC-Pro

We were interested to determine if this *Drosophila* cell system could be used as a screen for gene silencing suppression effects caused by other virus proteins. It has been suggested that the ORF0

protein of potato leafroll virus (PLRV) may act as a suppressor of gene silencing (REF). *Drosophila* cells transfected an ORF0 expression plasmid along with the *lacZ* expression plasmid and dsRNA demonstrated suppression of gene silencing compared to cells transfected with the *lacZ* expression plasmid and dsRNA alone (Fig 11). This identification of gene silencing suppression with the PLRV ORF 0 suggests that this system will be a useful screening tool to identify other proteins that have similar functions.

The observations here that plant virus proteins can suppress gene silencing in *Drosophila* cells indicates that at least part of the pathway of PTGS is conserved between plants and *Drosophila*. The *Drosophila* cell system has been useful for elucidating some of the biochemical detail of PTGS and a nuclease activity apparently deriving sequence-specificity from essential ~25 nucleotide RNA species has been identified (Hammond et al, 2000).

Suppressors of PTGS that function in *Drosophila* cells will be useful for further dissection of the mechanisms of PTGS in *Drosophila* cells as well as being an amenable system for study of the mode of action of the plant virus proteins themselves. The *Drosophila* system will also be a good starting point for the identification of proteins with which the suppressor proteins interact.

Plasmid constructions.

A region (nts 1055-2449) of the TEV genome containing the HCPRO sequence was amplified by Polymerase chain reaction (PCR) using primers HCPRO-1 (5'-CC**GGTACC**ATGAGCGACAAATCAATCTCTGAGGC-3') (SEQ ID No: 3) and HCPRO-2 (5'- GG**CTCGAG**CTACACATCTCGGTTCATCCCTCC-3') (SEQ ID No: 4). Primer HCPRO-1 contains a KpnI site (shown in bold in the sequence) and an ATG initiation codon (shown underlined) in addition to the TEV sequence. Primer HCPRO-2 contains an XhoI site (shown in bold) and the complement of a TAG termination codon (shown underlined) in addition to the TEV sequence. The PCR product was cloned into pGEM-Teasy (Promega) and then subcloned as a KpnI-XhoI fragment into pMT/V5-HisA (Invitrogen) cut with KpnI and XhoI to give plasmid pMT-HCPRO.

PLRV ORF0 construction

The PLRV ORF0 sequence was amplified by PCR using cloned cDNA as a template and primers 499 (5'-ATAGCCCATGGTTGTATTGACCC-3') (SEQ ID No: 5) and 500 (5'-TTCCAGGTACCTCTCATTCTTGTAATTCC-3') (SEQ ID No: 6) to introduce flanking *NcoI* and *KpnI* sites into the PCR product. The PCR product was cloned into pMT/V5-HisA to produce plasmid pMT-ORF0.

RNA synthesis. cDNA corresponding to ~500bp of the 5' end of the LacZ gene was amplified using pcDNA3.1/HisB/lacZ as a template and primers LacZ-1 (5'-TAATACGACTCACTATAGGGAGACCCAAGCTGGCTAGC-3') (SEQ

ID No: 7) and LacZ-2 (5'-TAATACGACTCACTATAGGGCAAACGGCGGATTGACCG-3') (SEQ ID No: 8). Both primers contained T7 RNA polymerase initiation sequences (shown underlined). The PCR product was used to direct synthesis of dsRNA using T7 RNA polymerase (Invitrogen) after which the DNA template was removed by DNase digestion.

Cell culture and Transfection. DS2 cells and DES expression medium were part of the *Drosophila* Expression System (Invitrogen) and cells were grown according to the manufacturer's instructions. Cells were grown in 60mm dishes and transfected with 10µg plasmid DNA either alone or with 5 µg dsRNA by calcium phosphate co-precipitation. After transfection the cells were washed twice in DES medium and grown for eight hours before expression of proteins was induced by addition of $CuSO_4$. A stably transformed line expressing the HCPRO gene was established by co-transfection of cells with the relevant plasmid and pCo-Hygro (Invitrogen) followed by selection of transformed cells in medium containing hygromycin. Cells were stained to detect *lacZ* gene expression using a β-Gal Staining Kit (Invitrogen) 48hrs after transfection.

EXAMPLE 3: Groundnut Rosette Virus (GRV) ORF3 Suppresses RNA Interference in Drosophilla Cells

The GRV ORF3 sequence was amplified by PCR using cloned cDNA as a template and two sets of primers.

The first set of primers, GRV3HTFOR (5'-CGATGGTACCACAATGGACACCACCC-3') (SEQ ID No: 9) and GRV3MTHREV (5'-CGATCTCGAGTCAATGGTGATGGTGATGATGCCACTTATTGGCAGCGG-3') (SEQ ID No: 10), introduce a polyhistidine tag at the carboxy-terminal end of the ORF3 protein and flanking *KpnI* and *XhoI* sites. This PCR product was cloned into pMT/V5-HisC (Invitrogen) to produce plasmid pMT-ORF3/His. The second set of primers, GRV3MTHFOR (5'-CGATGGTACCACAATGGGACATCATCACCATCACCATGACACCACCCCGG-3') (SEQ ID No: 11) and GRF3HTREV (5'-CGATCTCGAGTCACCACTTATTGGCAGCGG-3') (SEQ ID No: 12), introduce a polyhistidine tag at the amino-terminal end of the ORF3 protein and flanking *KpnI* and *XhoI* sites. This PCR product was cloned into pMT/V5-HisC to produce plasmid pMT-His/ORF3.

Drosophila (DS2) cells were grown in Schneider's *Drosophila* medium (Life Technologies). Stably transformed *Drosophila* (DS2) cell lines expressing the modified ORF3 proteins were produced by co-transfection of cells with either pMT-ORF3/His or pMT-His/ORF3 along with pCo-Hygro (Invitrogen) using calcium phosphate co-precipitation, followed by selection of transformed cells in medium containing 300 µg/ml hygromycin. Expression of the modified ORF3 proteins was confirmed by immunoblotting using an anti-6His antibody (Sigma). The cell lines were called DS2-ORF3/His (expressing pMT-ORF3/His) and DS2-His/ORF3 (expressing pMT-His/ORF3).' Cultures of control DS2 cells and of both transformed cell lines

expressing the modified ORF3 proteins were transfected by calcium phosphate co-precipitation in 60mm tissue culture dishes with either 10 µg of pMT/V5-His/lacZ (Invitrogen) or 10 µg of pMT/V5-His/lacZ and 5 µg of double-stranded (ds) RNA corresponding to the 5'-terminal 500 nucleotides of the *lacZ* gene to induce gene silencing. Cells were stained to detect *lacZ* gene expression using a β-gal staining kit (Invitrogen) 48 hours after transfection. The results are shown in Table 1.

Transfection efficiencies were determined in the cultures transfected with pMT/V5-His/lacZ and were approximately 55% for all three cell types. Only 10.25% of control DS2 cells transfected with pMT/V5-His/*lacZ* + dsRNA stained for *lacZ* expression, representing 19.2% of the cells staining when the cells were transfected with pMT/V5-His/*lacZ* alone, and indicating that RNA interference (gene silencing) was occurring. In contrast, 30.75% of DS2-ORF3/His cells and 33% of DS2-His/ORF3 cells transfected with pMT/V5-His/*lacZ* + dsRNA stained for *lacZ* expression representing 54.9% and 63% of the cells staining when the cells were transfected with pMT/V5-His/*lacZ* alone respectively. This indicates that RNA interference (gene silencing) was suppressed in the *Drosophila* cell lines expressing the modified versions of the ORF3 protein and shows that the ORF3 protein can suppress RNA interference (gene silencing) in heterologous systems.

Cell Type	Transfection	% cells stained *
DS2	pMT/V5-His/*lacZ*	53.38
DS2	pMT/V5-His/*lacZ* + dsRNA	10.25
DS2-ORF3/His	pMT/V5-His/*lacZ*	56
DS2-ORF3/His	pMT/V5-His/*lacZ* + dsRNA	30.75
DS2-His/ORF3	pMT/V5-His/*lacZ*	53.4
DS2-His/ORF3	pMT/V5-His/*lacZ* + dsRNA	33

Table 1. Effects of GRV ORF3 on RNA interference in *Drosophila* cells. *Four randomly selected fields of view each containing ~100 cells were selected in each of duplicate plates and the number of cells staining blue was counted for each experiment.

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CLAIMS

1. A method of suppressing or inhibiting one or more gene silencing mechanisms in a non-plant host cell through the use of a plant virus protein exhibiting post-transcriptional gene silencing suppressing functions.

2. A method as claimed in Claim 1 wherein the expression of a heterologous protein encoded by a virus vector or transgene in the non-plant host cell is increased.

3. A method as claimed in Claim 1 wherein expression of a heterologous protein encoded by a virus vector or transgene in the non-plant host cell is protected from post-transcriptional gene silencing effected by the host cell.

4. A method as claimed in any one of Claims 1 to 3 wherein the non-plant cell is an animal cell.

5. A method as claimed in any one of Claims 1 to 4 wherein the non-plant cell is a mammalian cell.

6. A method as claimed in any one of Claims 1 to 4 wherein the non-plant cell is an insect cell.

7. The method as claimed in Claim 5 wherein said host cell is a *Drosophila* cell.

8. The method as claimed in any one of Claims 1 and 7 wherein said viral vector encoding said heterologous protein is based on PVX, PVY, or TMV.

9. The method as claimed in any one of Claims 2 and 8, wherein a transgenic host cell expressing said plant virus protein is inoculated with a viral vector encoding the heterologous protein of interest.

10. The method as claimed in any one of Claims 2 and 9, wherein a transgenic host cell expressing a heterologous protein of interest is inoculated with a viral vector encoding the plant virus protein.

11. The method as claimed in any one of Claims 2 to 10 wherein said plant virus protein and said heterologous protein of interest are encoded by a single viral vector.

12. The method as claimed in any one of Claims 1 to 11 wherein said plant virus protein is a cysteine-rich plant virus protein.

13. The method as claimed in any one of Claims 1 to 11 wherein said plant virus protein is the HC-Pro protein of *tobacco etch virus* (TEV),

the 2b protein of *cucumber mosaic virus* (CMV), the ORF0 protein of *potato leafroll virus* (PLRV), the 16K CRP protein of the *tobacco rattle virus*, the 12K CRP of *Pea early browning virus* or is a functional equivalent of these proteins.

14. The use of a plant virus protein as a suppressor of the gene silencing mechanism of a non-plant host cell.

15. The use as claimed in Claim 14 wherein said plant virus protein is a cysteine-rich plant virus protein.

16. The use as claimed in Claim 14 wherein said plant virus protein is the HC-Pro protein of *tobacco etch virus* (TEV), the 2b protein of *cucumber mosaic virus* (CMV), the ORF0 protein of *potato leafroll virus* (PLRV), the 16K CRP protein of the *tobacco rattle virus*, the 12K CRP of *Pea early browning virus* or is a functional equivalent of these proteins.

17. A vector comprising a polynucleotide sequence which encodes at least one plant virus protein displaying post-transcriptional gene suppressor activity and which is capable of expressing said polynucleotide sequence in a non-plant host cell.

18. A vector as claimed in Claim 17 wherein the post-transcriptional gene suppressor protein is derived from the HC-Pro protein of *tobacco etch virus* (TEV), the 2b protein of *cucumber mosaic virus* (CMV),the ORF0 protein of *potato leafroll virus* (PLRV), the 16K CRP protein of the *tobacco rattle virus*, the 12K CRP of *Pea early browning virus* or is a functional equivalent of these proteins.

19. The use of a cysteine-rich plant virus protein displaying post-transcriptional gene silencing as a suppressor of the gene silencing mechanism of a host cell.

20. The use as claimed in Claim 19 wherein said plant virus protein is the 16K CRP of tobacco rattle virus or the 12K CRP of pea early browning virus.

21. A method of suppressing or inhibiting one or more gene silencing mechanisms in a host cell through the use of a cysteine-rich plant virus protein exhibiting post-transcriptional gene silencing suppressing functions.

22. The method as claimed in Claim 19 wherein said plant virus protein is the 16K CRP of tobacco rattle virus or the 12K CRP of pea early browning virus.

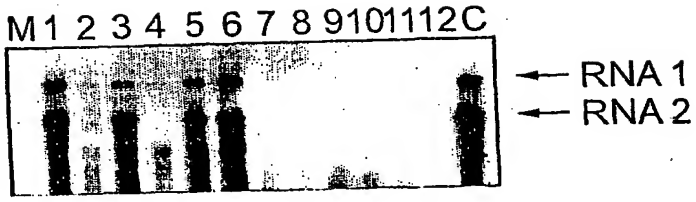

Fig. 1a

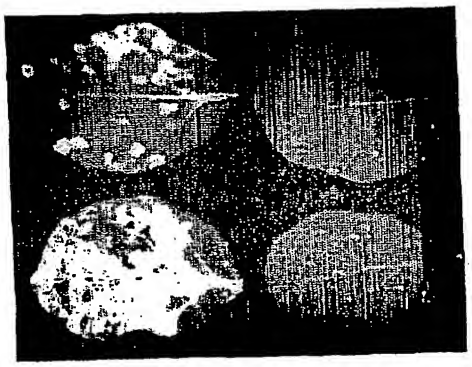

Fig. 1b

```
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PEBV12K MKCAVSTCEVEAOSN-KFTCSMKCANKYNRHLAEKY
          *  *   *     *  ***   ***      *

TRV16K  GVTRRCAENNCGWF-VCVVINDFTFDVYNCCGRSHL
PEBV12K SIKRKCECVNCGWYPAIEVRADF-IEVYFCCGMKHL
          *  *   ****     *   **   ** *** **

TRV16K  EKCRKRVETRNREIWKQIRRNQAENMSATAKKSHNS
PEBV12K S----------------KVISSN----------
                          *  *  *

TRV16K  KTSKKKFKEDREFGTPKRFLRDDVPFGIDRLFAF
PEBV12K ---T-PKRKE-R-LNSPKRLFRDDIDFGLTGLFNESC
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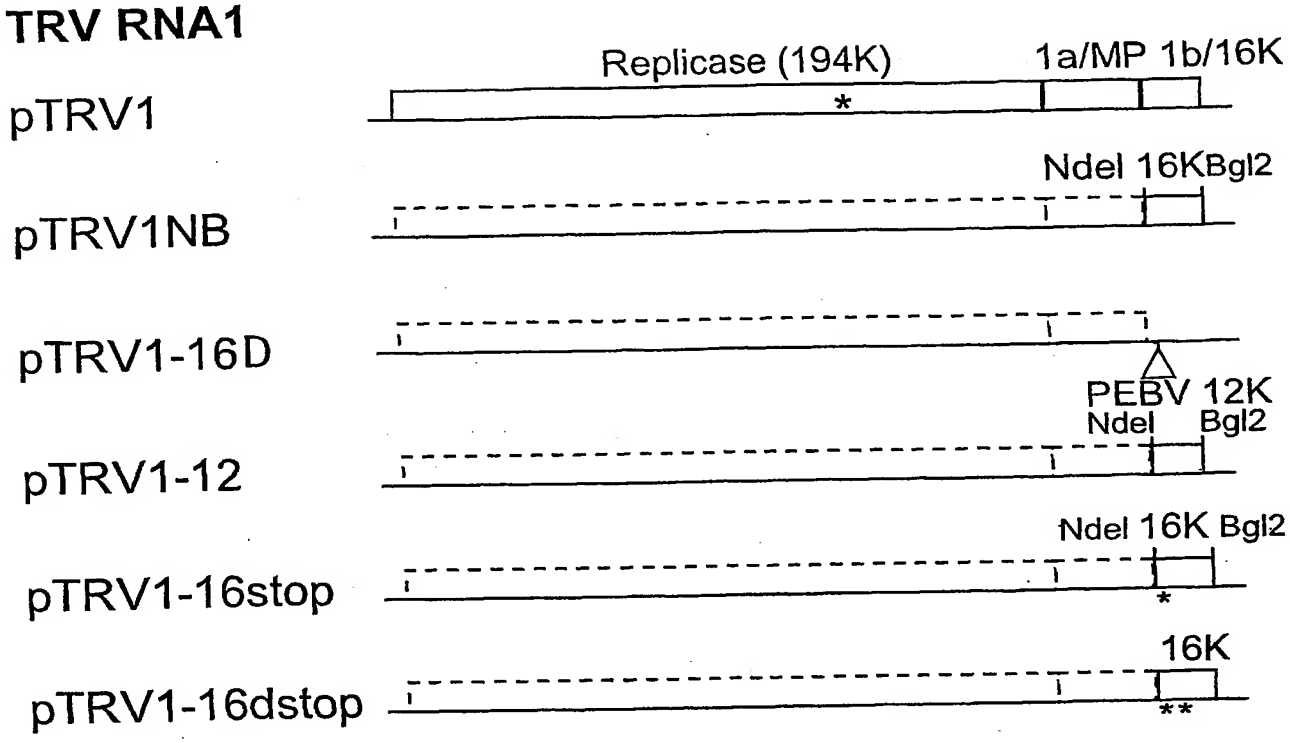
Fig. 2a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Replicase (194K)
1a/MP 1b/16K
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Ndel 16K Bgl2
pTRV1-16D
PEBV 12K
Ndel Bgl2
pTRV1-12
Ndel 16K Bgl2
pTRV1-16stop
*
16K
pTRV1-16dstop
**
TRV RNA2
pK20-RNA2
CP
2b
2c
pK20-GFPc
CP
GFP
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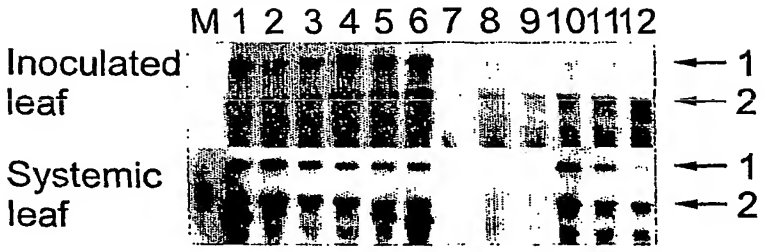
CP
CMV 2b

Fig. 2b



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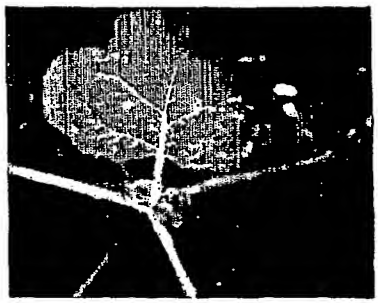


Fig. 3b

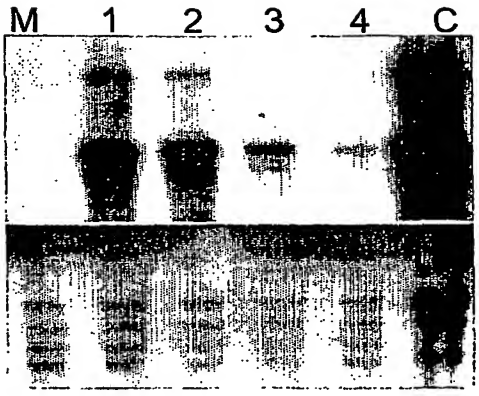


Fig. 3c

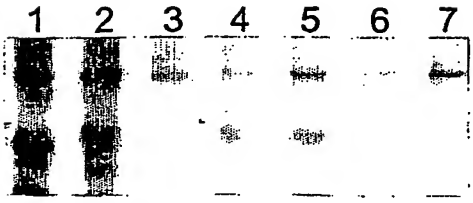


Fig. 3d

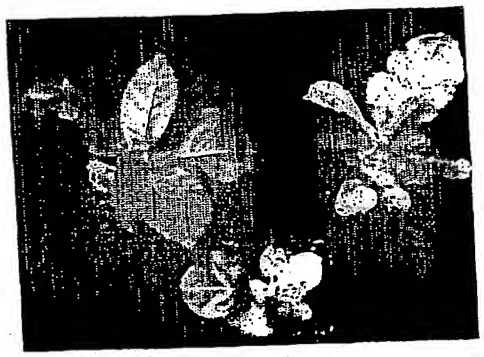


Fig. 4a

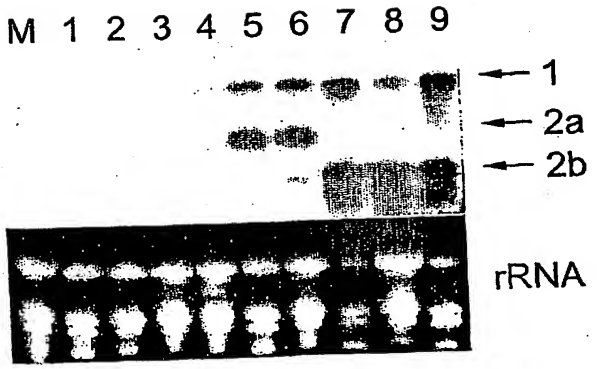
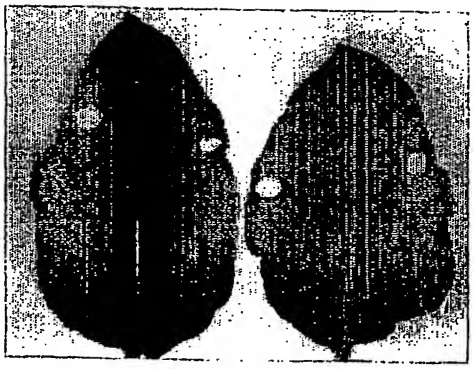


Fig. 4b



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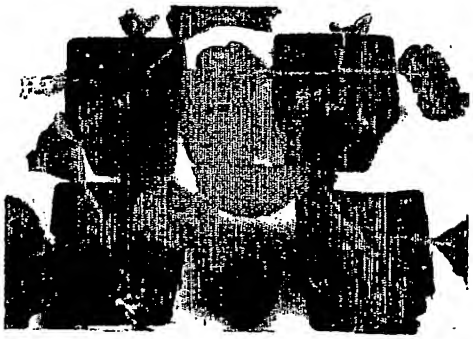

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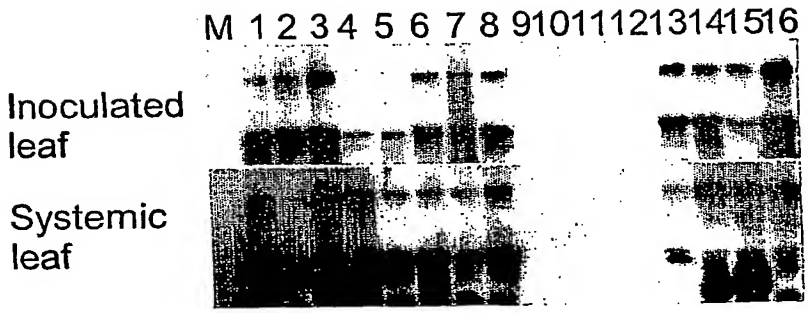
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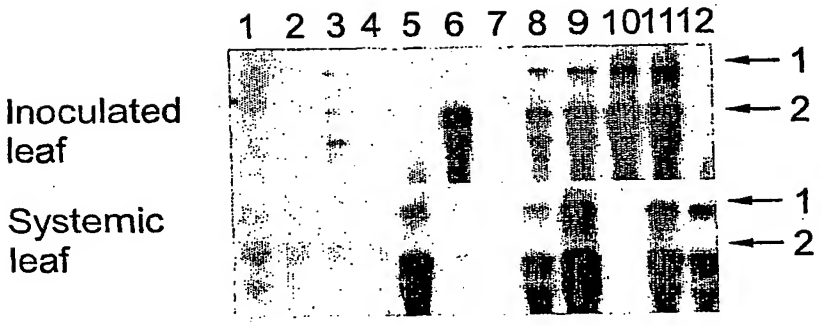
Inoculated leaf
Systemic leaf

Fig. 6a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Inoculated leaf
Systemic leaf
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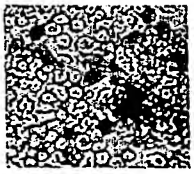
2
1
2

Fig. 6b



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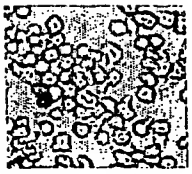

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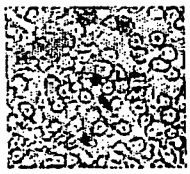


Fig. 7c

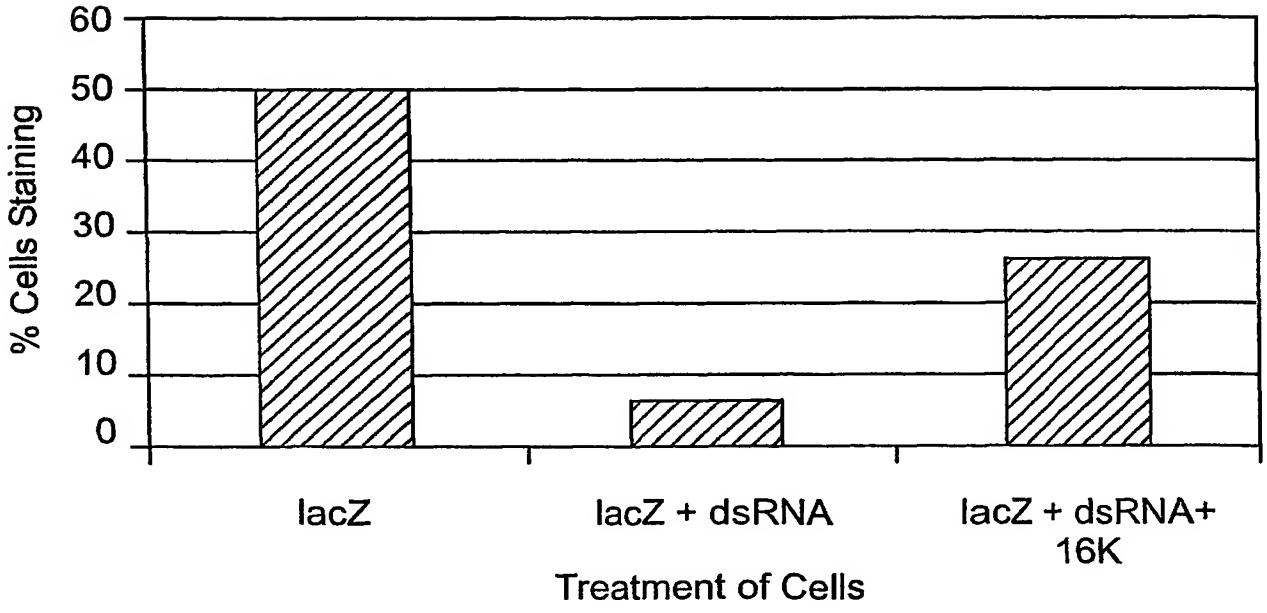
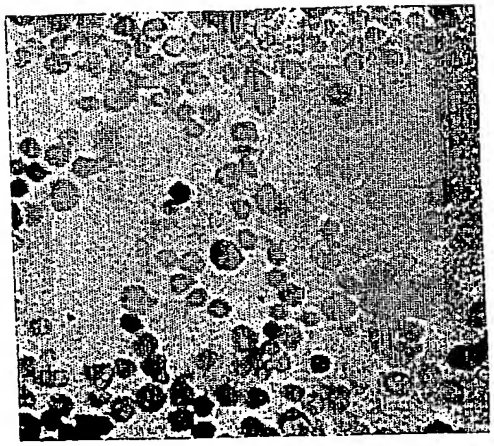


Fig. 7d

Fig. 8c



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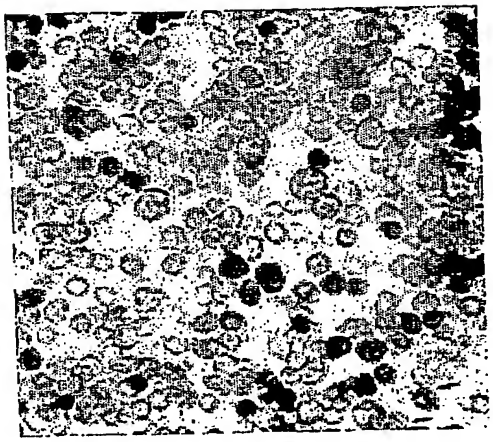
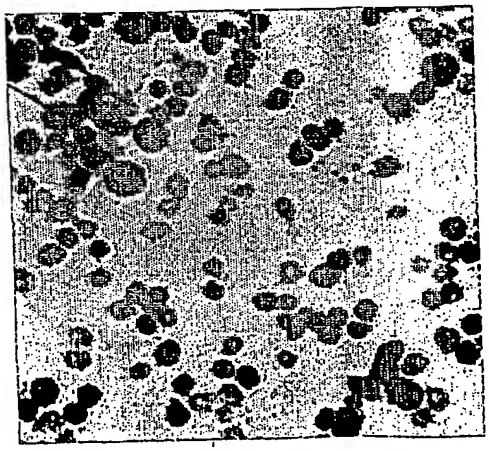
+HC-Pro

Fig. 8b



+ lacZ
+dsRNA

Fig. 8a



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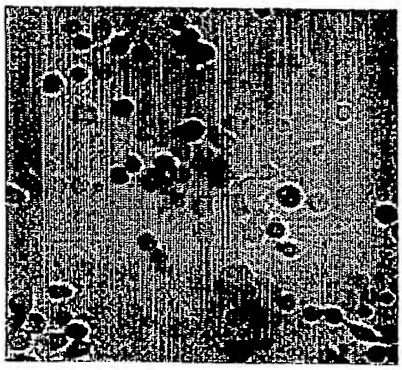
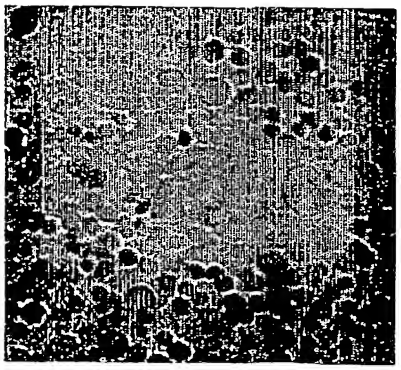


Fig. 9c + lacZ

Fig. 9d + lacZ +dsRNA



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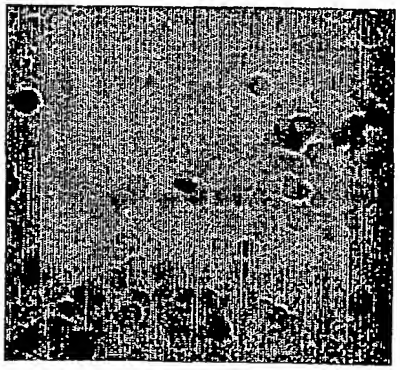
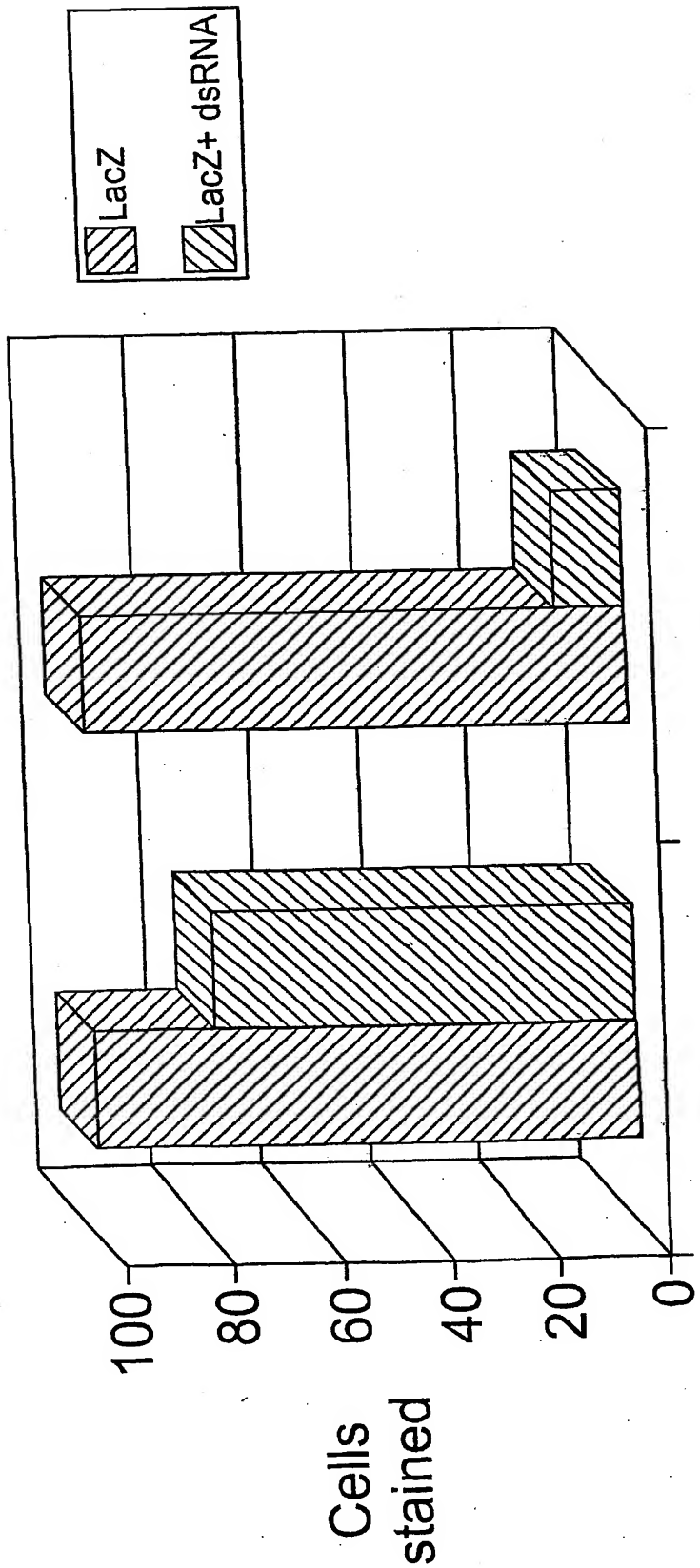


Fig. 9b + lacZ +dsRNA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DS2-VCL

Fig. 10

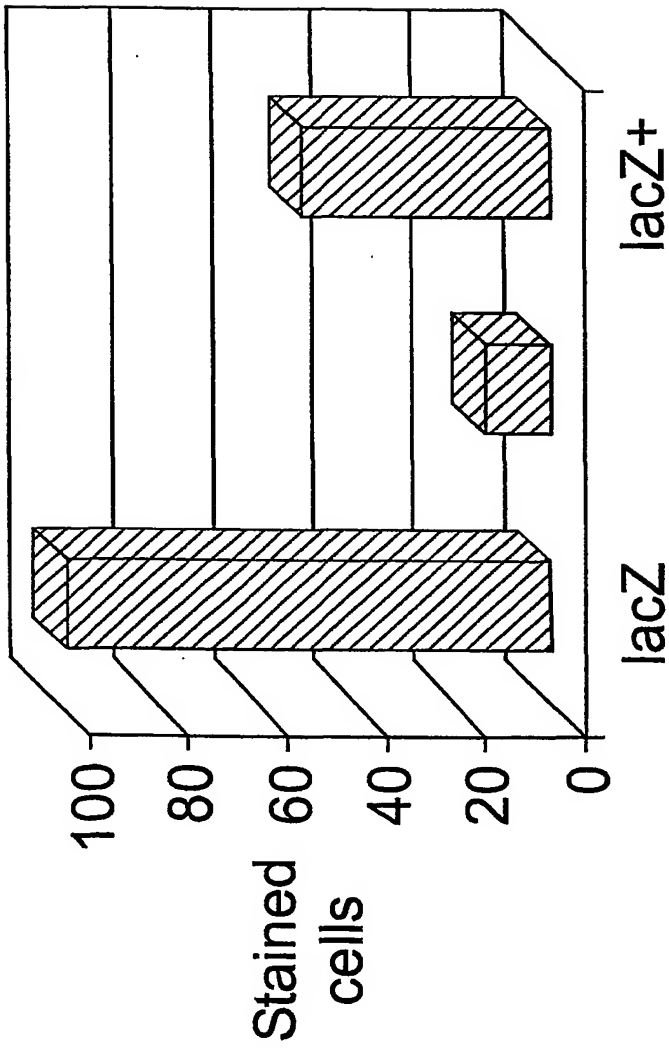

Fig. 11

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taatacgact cactataggg caaacggcgg attgaccg 38

<210> 7
<211> 141
<212> PRT
<213> Tobacco rattle virus

<400> 7

Met Thr Cys Val Leu Lys Gly Cys Val Asn Glu Val Thr Val Leu Gly
1 5 10 15

His Glu Thr Cys Ser Ile Gly His Ala Asn Lys Leu Arg Lys Gln Val
20 25 30

Ala Gln Met Val Gly Val Thr Arg Arg Cys Ala Glu Asn Asn Cys Gly
35 40 45

Trp Phe Val Cys Val Val Ile Asn Asp Phe Thr Phe Asp Val Tyr Asn
50 55 60

Cys Cys Gly Arg Ser His Leu Glu Lys Cys Arg Lys Arg Val Glu Thr
65 70 75 80

Arg Asn Arg Glu Ile Trp Lys Gln Ile Arg Arg Asn Gln Ala Glu Asn
85 90 95

Met Ser Ala Thr Ala Lys Lys Ser His Asn Ser Lys Thr Ser Lys Lys
100 105 110

Lys Phe Lys Glu Asp Arg Glu Phe Gly Thr Pro Lys Arg Phe Leu Arg
115 120 125

Asp Asp Val Pro Phe Gly Ile Asp Arg Leu Phe Ala Phe
130 135 140

<210> 8
<211> 107
<212> PRT
<213> Pea early browning virus

<400> 8

Met Lys Cys Ala Val Ser Thr Cys Glu Val Glu Ala Asp Ser Asn Lys
1 5 10 15

Phe Thr Cys Ser Met Lys Cys Ala Asn Lys Tyr Asn Arg His Leu Ala
20 25 30

Glu Lys Tyr Ser Ile Lys Arg Lys Cys Glu Cys Val Asn Cys Gly Trp
35 40 45

Tyr Pro Ala Ile Glu Val Arg Ala Asp Phe Ile Glu Val Tyr Phe Cys
50 55 60

Cys Gly Met Lys His Leu Ser Lys Val Ile Ser Ser Asn Pro Lys Arg
65 70 75 80

Lys Glu Arg Leu Asn Ser Pro Lys Arg Leu Phe Arg Asp Asp Ile Asp
85 90 95

Phe Cys Leu Thr Gly Leu Phe Asn Glu Ser Cys
100 105